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1. Holland et al. Anal. Biochem. 1994, 222(2), pp. 516-518.
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An *in Vitro* 96-Well Plate Assay of the Mitogen-Activated Protein Kinase Cascade

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Mitogen-activated protein (MAP) kinases of the extracellular signal-regulated kinase (ERK) family are activated in response to many growth and differentiation factors as well as some oncogenes. ERK activation follows phosphorylation by a class of specific upstream MAP kinase/ERK kinase (MEK) exemplified by MEK-1. Activated ERKs control many short- and long-term changes in cell function through phosphorylating a number of intracellular target substrates which include stathmin, a phosphoprotein regulating microtubule stability. We report here the development of a simple, 96-well plate, quantitative *in vitro* assay measuring purified ERK2 catalytic activation by a constitutive MEK-1 mutant (S218E S222E). Enzymatic activity was detected by ³²P phosphorylation of purified biotinylated stathmin captured on streptavidin-coated scintillation proximity assay beads which eliminates the need for wash steps. The assay was optimized and the $K_{0.5}$ value for ATP was found to be 0.9 μ M and the K_m for stathmin was determined to be 16 μ M. The assay was also used to determine IC_{50} values for the protein kinase inhibitors PD98059 and staurosporine. This simple assay allows several hundred quantitative measurements of MEK1-dependent ERK2 activation to be performed in a day. © 1999 Academic Press

Cell surface receptors for many cytokines, growth and differentiation factors as well as several oncogenes are linked to activation of a kinase cascade leading to

the extracellular signal-regulated kinase (ERK)² family of mitogen-activated protein (MAP) kinases (1–4). Two ERK gene products have been characterized in some detail (ERK1, ERK2) and their activation was found to be unusual insofar that it requires phosphorylation on both a Thr and Tyr residue located within the motif TXY of kinase domain VIII (Thr 183 and Tyr 185 of ERK2). This reaction is catalyzed by members of a dual specificity kinase family exemplified by ERK activator MAP kinase/ERK kinase-1 (MEK-1) which is in turn activated following its phosphorylation at Ser218 and Ser222 by the upstream kinase c-Raf-1 (1–4). Activated ERKs are known to phosphorylate a number of substrates including transcription factors, nuclear receptors, additional kinases, and cytoskeletal regulatory proteins (1, 4–8). One of these target substrates is stathmin, a phosphoprotein known to regulate cell microtubule stability (9, 10). As suggested by this range of phosphorylation targets, recent studies with a small molecule inhibitor of MEK-1 as well as use of dominant active or inhibitory MEK-1 mutants indicate a key role for ERKs in controlling diverse cell functions. These include cellular mitogenesis and oncogenic transformation, neuronal differentiation and survival, as well as molecular events regulating underlying memory and learning (1, 11–14). There is now an urgent need for new direct regulators of ERK activation state to provide essential new tools to facilitate further our understanding of the roles of this MAP kinase pathway in normal and pathological cell func-

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² Abbreviations used: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase; SPA, scintillation proximity assay; DTT, dithiothreitol; MBP, myelin basic protein; Mops, 4-morpholinepropanesulfonic acid; $K_{0.5}$, the substrate concentration which gives half-maximal velocity in the cascade assay.

tion. Such modulators could be diverse in nature but include new small molecule inhibitors of kinases in the ERK signaling cascade or, alternatively, novel dual specificity phosphatases mediating potent and selective dephosphorylation of activated ERKs.

One essential tool for the discovery and characterization of new MAP kinase regulators is a simple and inexpensive assay for quantitative determination of ERK catalytic activity. To meet this need we have developed an *in vitro* cascade kinase assay using [γ - 32 P]ATP and designed to run in a 96-well plate format where a purified constitutive MEK-1 mutant (S218E S222E) activates ERK2 to phosphorylate its natural substrate stathmin.

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP and streptavidin-coated scintillation proximity assay (SPA) beads (RPNQ 007) were from Amersham, England. PD98059 [2'-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] was purchased from Calbiochem and staurosporine was from LC Laboratories, U.S.A. Myelin basic protein was from Sigma, U.S.A., Glutathione-Sepharose was from Pharmacia, Sweden, sulfo-NHS-LC-biotin was from Pierce, U.S.A., and the 96-well plates (PET) were from Wallac, Finland.

Preparation of ERK2

Mouse ERK2 was overexpressed as a GST fusion protein in *Escherichia coli* (15). Cells were disrupted in the French press at a cell pressure of 16,000 psi and the fusion protein was isolated from the 10,000 g supernatant. The protein was bound to a glutathione-Sepharose column which was washed and from which ERK2 was eluted by incubation with thrombin. The fusion protein has a thrombin cleavage site between GST and ERK2. The eluted protein was approximately 80% pure and was further purified on FPLC Mono Q. This gave an apparent homogeneous preparation as judged from SDS-PAGE. The sample was dialyzed against 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 2 mM DTT, and 0.5 mM EGTA. To the dialyzed sample was added 50 % glycerol and it was stored at -20°C at a concentration of 1.8 mg/ml.

Preparation of MEK-1 (S218E S222E)

A MEK-1 mutant was constructed by replacing two serine residues (Ser 218 Ser 222) by aspartic acid (MEK-EE). These are two critical residues which trigger enzymatic activation following their phosphorylation by Raf and lead to constitute activation upon mutation to aspartate or glutamate (16, 17). The MEK-EE

was expressed in *E. coli* as a fusion protein with GST at the N-terminus and a His-tag at the C-terminus. The cells were broken in the French press at a cell pressure of 16,000 psi and the protein was isolated from the 10,000 g supernatant. The sample was applied to a Ni-agarose column, the column was washed, and MEK-EE was eluted with 200 mM imidazol. The eluted protein was then applied to a glutathione-Sepharose column and MEK-EE eluted by cleavage with factor X. The protein construct has a factor X cleavage site between GST and MEK-EE. The eluted sample was once again applied to a Ni-agarose column and eluted with imidazol. This sample was over 95% pure on SDS-PAGE. The sample was dialyzed against 50 mM Tris, pH 8.0, containing 10 mM 2-mercaptoethanol, 5% glycerol, 0.02% Triton X-100, 4 mM MgCl₂, 100 mM NaCl, and 1 mM EGTA and stored at -80°C at a concentration of 0.3 mg/ml.

Preparation of Stathmin

Human stathmin was expressed in *E. coli*, cells were disrupted using a French press at 16,000 psi and the protein was isolated from the 10,000 g supernatant. The supernatant was applied to a Q-Sepharose column in 50 mM Tris-HCl, pH 8.0, and stathmin was eluted with a linear NaCl gradient. Fractions containing stathmin were further purified on hydroxylapatite followed by phenyl-Sepharose. As the final purification step the stathmin pool from phenyl-Sepharose was applied to FPLC Mono Q. The pool from the Mono Q column gave a homogenous protein on SDS-PAGE and the protein was over 98% pure on reverse-phase HPLC. The protein was dialyzed against 20 mM Tris-HCl, pH 7.5, and stored at -80°C at a concentration of 4.0 mg/ml.

Biotinylation of the Stathmin

For biotinylation, stathmin (3 mg/ml) was first dialyzed against PBS after which a 1.5 molar excess of sulfo-NHS-LC-biotin (Pierce) was added followed by incubation at 4°C for 2 h. The sample was then dialyzed against PBS and finally against 20 mM Tris-HCl, pH 7.5. The sample was stored at -80°C at a concentration of 2.0 mg/ml. Under these conditions 1.2 biotin molecules were incorporated per stathmin molecule. To ensure that the biotinylation did not affect the phosphorylation of stathmin we assayed biotinylated and nonbiotinylated stathmin in a filter assay (18). No difference in incorporation of 32 P into biotinylated and nonbiotinylated stathmin was detected. However, when biotin was incorporated at a higher molar ratio, between 4 and 9 biotin molecules per stathmin molecule, the incorporation of 32 P decreased. Myelin

basic protein (MBP) was biotinylated following the same protocol as described above.

ERK MAP Kinase Cascade Assay

The assay was performed in 96-well plates. Assay solutions and plates were pre-incubated at 37°C for 5 min. To each well we added 40 μ l of assay buffer (18.7 mM Mops, pH 7.0, 12.5 mM MgCl_2 , 0.62 mM EGTA, 62.5 μ M NaF, 1.25 mM DTT, and 3.75 μ M ATP, 3.1 μ Ci/ml [γ - 32 P]ATP) and 10 μ l of kinase/substrate mix containing 1.2 μ M ERK2, 300 nM MEK-EE, and 5 μ M biotinylated stathmin in 10 mM Mops at pH 7.0. The final concentrations in the assay were 15 mM Mops, pH 7.0, 10 mM MgCl_2 , 0.5 mM EGTA, 50 μ M NaF, 1 mM DTT, 240 nM ERK2, 60 nM MEK-EE, 3 μ M ATP with 125 nCi [γ - 32 P]ATP, and 1 μ M biotinylated stathmin. The plates were incubated at 37°C for 45 min. At the end of the incubation 200 μ l of SPA bead solution (PBS containing 0.1% Triton X-100, 5 mM EDTA, 50 μ M ATP, 2.5 mg/ml streptavidin-coated SPA beads) was added per well. Two hundred microliters of this solution contained 0.5 mg of beads with a binding capacity of at least 50 pmol of biotinylated stathmin. After addition of the beads the plates were left to incubate for 1 h at room temperature. To reduce the background the plates were centrifuged at 3000 rpm (1800g) for 5 min before they were counted in a 1450 MicrobetaPlus liquid scintillation counter (Wallac). Alternatively, the plates were left overnight for the beads to sediment by gravity.

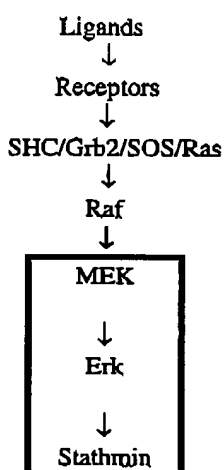


FIG. 1. Schematic representation of signaling leading to ERK MAP kinase activation. ERK activation is triggered through phosphorylation by MEK-1, which in turn is phosphorylated and activated by kinases of the Raf family. The *in vitro* kinase assay described here recreates the final steps (boxed) of the signaling pathway.

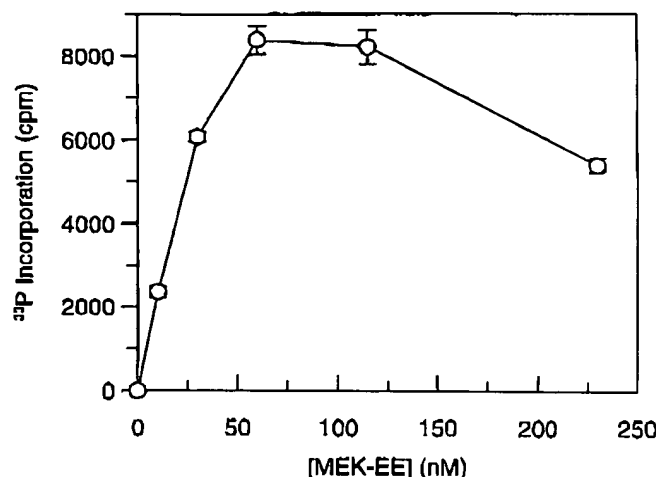


FIG. 2. MEK concentration dependence. Assays were performed as described under Materials and Methods with the exception that the ERK2 concentration was kept constant at 188 nM and the concentration of MEK-EE was varied between 10 and 230 nM. After 45 min incubation at 37°C the reaction was stopped by addition of 200 μ l bead solution. After 1 h incubation at room temperature the beads were sedimented by centrifugation at 3000 rpm for 5 min and subsequently counted in a 96-well plate scintillation counter. Each data point is the average of three determinations \pm SD.

RESULTS AND DISCUSSION

Activation of ERK family MAP kinases is an important event triggered by several signaling cascades. What is now required are new specific small molecule or enzymatic inhibitors of ERK activation to demonstrate definitively the function of this MAP kinase class particularly in various disease states. As a tool to help discover and characterize such inhibitory molecules we have developed an *in vitro* kinase cascade assay detecting MEK-1-dependent activation of ERK together with phosphorylation of its natural substrate stathmin. This *in vitro* assay recreates the final steps of a cascade common to many signaling pathways (Fig. 1).

The proteins used in this assay were all over 95% pure when analyzed by SDS-PAGE. The assay is performed in 96-well plates and is based on biotinylated stathmin bound to streptavidin-coated SPA beads. In the presence of [γ - 32 P]ATP, ERK-dependent phosphorylation of stathmin stimulates bead-encapsulated scintillant to emit light which can be detected in a scintillation counter. Free [γ - 32 P]ATP does not significantly stimulate the SPA beads, thereby eliminating the need for any wash steps or sample transfer. The background in the assay can however be reduced by centrifugation or sedimentation which separates the beads together with their associated 32 P-phosphorylated substrate from the bulk of unincorporated [γ - 32 P]ATP.

To establish optimal conditions for the assay the concentration-dependence of the kinases, MEK-1

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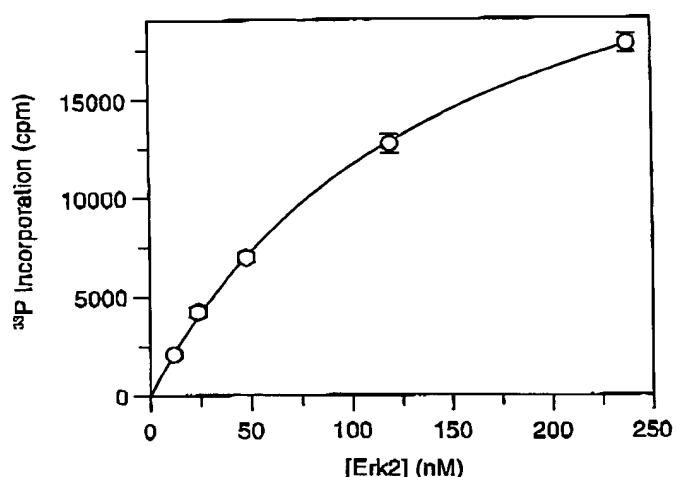


FIG. 3. ERK2 concentration dependence. Assays were performed as described under Materials and Methods with the exception that the MEK-EE concentration was kept constant at 60 nM and the concentration of ERK2 was varied between 24 and 240 nM. After 45 min incubation at 37°C the reaction was stopped by addition of 200 μ l bead solution. After 1 h incubation at room temperature the beads were sedimented by centrifugation at 3000 rpm for 5 min and subsequently counted in a 96-well plate scintillation counter. Each data point is the average of three determinations \pm SD.

(S218E S222E) and ERK2 were first determined. One of the kinases was kept at a constant concentration and the concentration of the second was varied. Figure 2 shows the incorporation of 32 P phosphate into stathmin when the ERK2 concentration was kept constant at 188 nM and the concentration of MEK-EE was varied between 10 and 230 nM. Optimal 32 P incorporation was reached at 60 nM MEK-EE. At concentrations above 100 nM the incorporation of phosphate was inhibited. To determine the concentration dependence of ERK2 in the cascade reaction, the MEK-EE concentration was kept constant at 60 nM and the ERK2 concentration was varied between 24 and 950 nM (Fig. 3). Optimal incorporation of 32 P phosphate to stathmin was reached at 240 nM. Importantly, when either MEK-EE or ERK2 was omitted from the reaction mixture, the incorporation of 32 P into stathmin was not significantly over background. Based on these results all subsequent assays were performed with 60 nM MEK-EE and 240 nM Erk2, unless otherwise indicated. These enzyme concentrations gave optimal phosphate incorporation into stathmin. The background in the assay was between 500 and 1200 cpm and appeared to vary slightly between batches of the beads. This gave a signal to noise ratio of between 8- and 20-fold.

To determine the time dependence of the kinase cascade reaction, standard reaction mixtures were incubated at 37°C. At various time points, between 5 min and 2 h, the reactions were stopped by addition of 200

μ l of the bead solution. The reaction showed linearity between 5 and 45 min (Fig. 4). A short lag phase was seen during the first 5 min. Since this lag phase was not present when preactivated ERK2 was used in the assay, it presumably reflects the time required for initial activation of ERK2 by MEK-1. To ensure conditions were chosen where 100% of biotinylated stathmin binds to the SPA beads, assays were performed with either 50 or 100 pmol of biotinylated stathmin. At the end of the incubation increasing amounts of SPA beads were added to the reactions. The streptavidin-coated beads have a binding capacity of 100 pmol/mg. The binding curves for the reactions containing 50 or 100 pmol of stathmin were essentially parallel up to 0.5 mg of added beads. No further increase in signal was seen after this point in assays containing 50 pmol stathmin, indicating maximal binding to beads. In the incubations with 100 pmol stathmin, addition of 1 mg of beads increases the captured signal by a factor of 2. These results give the expected stoichiometry and show that all stathmin from the reactions can be captured on beads. Reactions were routinely performed with 50 pmol stathmin.

To further characterize the cascade assay, the $K_{0.5}$ value for ATP and the K_m value for stathmin were next determined. From Lineweaver-Burk plots the $K_{0.5}$ value for ATP was determined as 0.9 μ M and the K_m value for stathmin was found to be 16 μ M (Fig. 5). MBP is a substrate for several protein kinases, including MAP kinase. For comparison with stathmin we also tested biotinylated MBP as substrate in the cascade

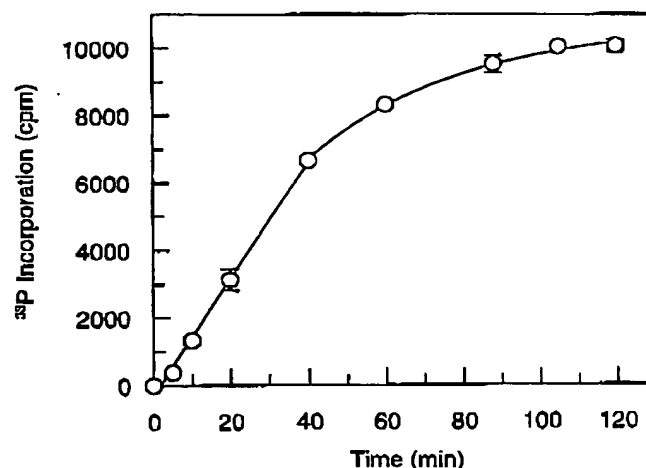


FIG. 4. Time dependence of the cascade assay. Reaction mixtures were incubated at 37°C as described under Materials and Methods. At various time points between 5 and 120 min the reactions were stopped by addition of 200 μ l bead solution. When the bead solution was added at time zero and the sample was incubated at 37°C for 120 min, the signal was not higher than background. Each data point is the average of three determinations \pm SD.

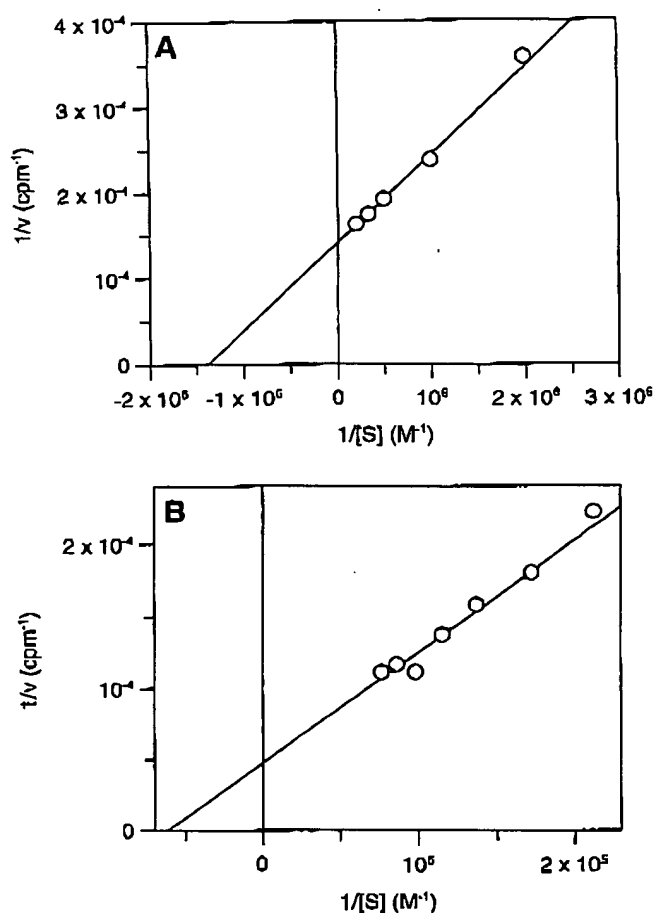


FIG. 5. Determination of the $K_{0.5}$ and K_m values for ATP and stathmin, respectively. Reactions were performed as described under Materials and Methods with the exception that the concentration of the substrate, ATP or stathmin, was varied. From the initial reaction velocity data Lineweaver-Burk plots were constructed and the $K_{0.5}$ and K_m values were determined. For ATP (A) the $K_{0.5}$ was determined as $0.9 \mu\text{M}$ and for stathmin (B) the K_m was determined as $16 \mu\text{M}$.

assay and the K_m was determined to be $20 \mu\text{M}$. This shows that components of the assay cascade can easily be exchanged to test for specificity and relative efficiency.

One possible use of this *in vitro* assay is to screen for small molecule inhibitors of the protein kinases within the cascade. To test its utility for this purpose we next determined the IC_{50} values for two known protein kinase inhibitors. First, PD98059 [2'-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] that has been shown to be a potent and highly selective inhibitor of MEK-1 (12), was tested at concentrations between 1×10^{-7} to $2 \times 10^{-4} \text{ M}$. From the inhibition curve half-maximal inhibition was observed at $20 \mu\text{M}$ (Fig. 6), a value in good agreement with the published IC_{50} value of approximately $10 \mu\text{M}$ (12). We also examined the

inhibition of the reaction cascade by the nonselective protein kinase inhibitor staurosporine, which displayed an IC_{50} of $0.15 \mu\text{M}$ (Fig. 7). Thus, the cascade assay can be used to detect inhibitors or other molecules interacting with and affecting the activity of the kinases in the signaling cascade.

In summary, we have developed a simple 96-well plate *in vitro* kinase cascade assay detecting MEK-1-dependent activation of ERK together with phosphorylation of its natural substrate stathmin. The assay employs $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and SPA-bead technology and allows handling of up to 1000 kinase reactions/day. We have shown that the assay readily detects kinase inhibition by small molecules, not only to ERK but also to the upstream kinase MEK-1. Thus, inhibitors to two kinases can be identified in one assay, although it is not possible to distinguish whether the compounds affect MEK-1, ERK-2, or both with this rapid screening procedure. Positive compounds can be tested subse-

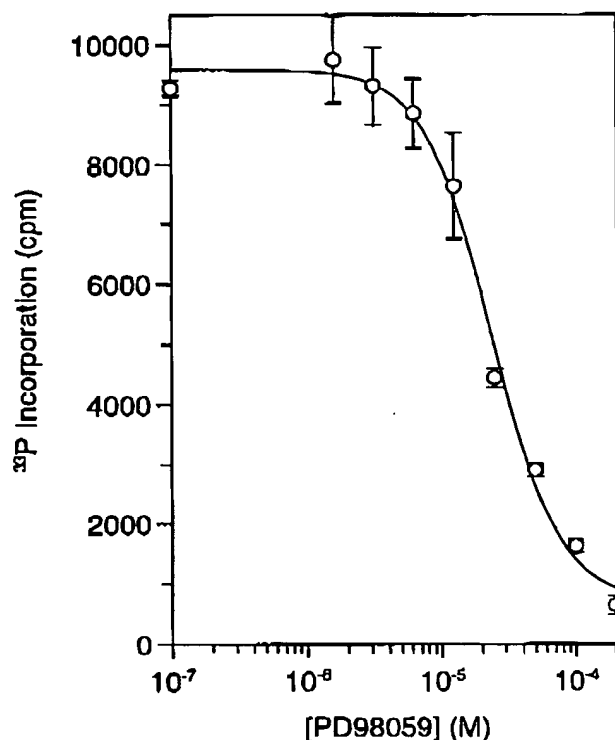


FIG. 6. Inhibition of the kinase cascade reaction by the MEK-specific inhibitor PD98059 [2'-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]. Reaction mixtures were incubated with increasing concentration of the inhibitor. After 45 min incubation at 37°C the reaction was stopped by addition of $200 \mu\text{l}$ bead solution. After 1 h incubation at room temperature the beads were sedimented by centrifugation at 3000 rpm for 5 min and subsequently counted in a 96-well plate scintillation counter. Each data point is the average of three determinations \pm SD. From the inhibition curve the IC_{50} value was calculated to be $20 \mu\text{M}$.

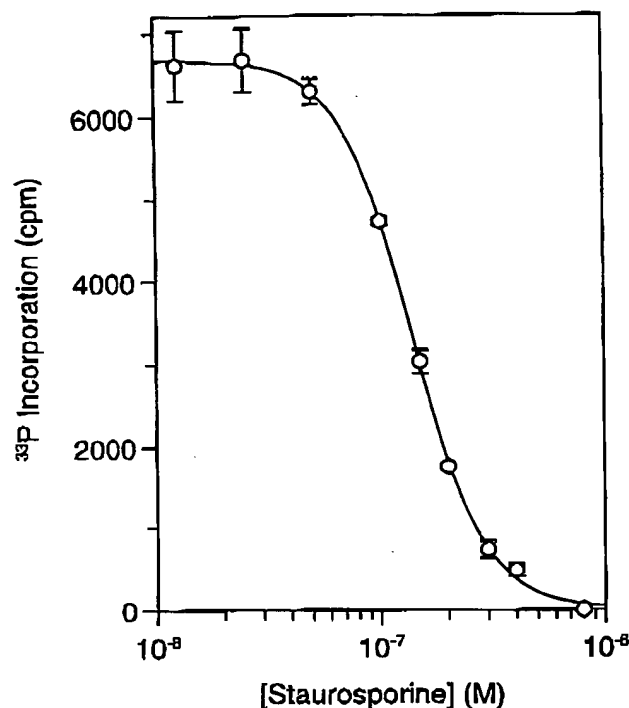


FIG. 7. Inhibition of the kinase cascade reaction by the nonspecific kinase inhibitor staurosporine. Reaction mixtures were incubated with increasing concentrations of staurosporine. After 45 min incubation at 37°C the reaction was stopped by addition of 200 μ l bead solution. After 1 h incubation at room temperature the beads were sedimented by centrifugation at 3000 rpm for 5 min with subsequent counting in a 96-well plate scintillation counter. Each data point is the average of three determinations \pm SD. The IC_{50} value was calculated to be 0.15 μ M.

quently for specificity in individual kinase assays. This will considerably reduce both the amounts of reagents used and time. This technique is of clear value for the detection of novel small molecule kinase inhibitors, although it also has clear utility in the characterization of protein regulators of MAP kinase activation such as members of the dual-specificity phosphatase family (20).

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